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Interaction of *Nocardia asteroides* at Different Phases of Growth with In Vitro-Maintained Macrophages Obtained from the Lungs of Normal and Immunized Rabbits

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The interactions of cells of *Nocardia asteroides* GUH-2 during different stages of growth with cultured macrophages obtained from the lungs of nonimmunized and immunized rabbits were studied. The nocardial cells from all stages grew intracellularly in "normal" alveolar macrophages; however, log-phase cells increased in numbers more rapidly than did stationary-phase cells. Macrophages obtained from the lungs of specifically immunized rabbits effectively inhibited the growth of stationary-phase cells but only temporarily retarded the growth of log-phase organisms. Specific antiserum added to the nocardial cells before incubation with presensitized macrophages caused enhanced phagocytosis and inactivation of the log-phase cells but had no effect on the fate of the stationary-phase nocardia. In addition, it was found that log-phase cells were phagocytized less effectively by normal macrophages than were the stationary-phase cells, and log-phase cells were more toxic to the macrophage monolayer. From these data we conclude that secondarily induced macrophages play a major role in host resistance to pulmonary nocardial infections, and antibody may be important for effective host resistance to the filamentous form of *N. asteroides*. Since the nocardia were able, with time, to overcome these effects, it appears that additional host factors (such as T-lymphocytes) must be involved in an effective host response to *N. asteroides*.

The initial events leading to pulmonary nocardiosis involve alveolar macrophage-nocardial cell interactions (5). It has been shown in vitro that alveolar macrophages obtained from rabbits not presensitized against *Nocardia* support the intracellular growth of virulent strains of *Nocardia asteroides* (3, 8, 9). Furthermore, it was observed that *N. asteroides* could initiate growth within the intact murine lung (5). Microscopic analysis of these lungs revealed that initially the nocardial cells were able to grow within alveolar macrophages, which resulted in acute bronchopulmonary infection. However, at approximately 72 h postinfection it was found that mice which survived this acute phase had initiated a secondary lung response that resulted in the complete elimination of nocardial cells from the "normal" murine lung 168 h after inoculation (5). Although polymorphonuclear neutrophils were present within the lesions in the lung, it appeared that macrophages and lymphocytes were most prominent during resolution of the infection (5). T-cell lymphocytes were important in this response since nude mice, which are T-cell deficient, were not able to effectively eliminate *N. asteroides* from the lung (5).

The specific ability of macrophages obtained from the lungs of presensitized animals to kill *N. asteroides* has not been evaluated. The role of specific antibody in alveolar macrophage-nocardia interactions has not been adequately assessed. Furthermore, the effect of nocardial culture age on these interactions has not been determined. This latter point is essential in attempting to understand the mechanisms of nocardial pathogenesis and host resistance since the phase of growth affects greatly the virulence of *N. asteroides* (7). The study reported herein utilizes in vitro maintained macrophages obtained from the lungs of normal and specifically presensitized rabbits in combination with autologous serum obtained from these animals in order to determine the roles of macrophages and antibody during the interaction with nocardial cells of different culture age.

MATERIALS AND METHODS

Microorganism. *N. asteroides* GUH-2 was isolated from a fatal human infection and maintained as previously described (6). Its growth cycle and pathogenicity for mice has been described (4-7). Single-cell suspensions of the organisms were prepared from

growing cultures in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) at 16 h (logarithmic phase), at 3 days (early stationary phase), and at 1 week (stationary phase) as previously described (1, 7).

Animals. New Zealand white rabbits, weighing approximately 2.3 to 2.7 kg, were obtained from Nitabell Rabbitry (Hayward, Calif.). They were used as the source of macrophages and serum.

Normal rabbit alveolar macrophages. Nonimmunized rabbits were sacrificed by injection of sodium pentobarbital into the marginal ear vein. The trachea was clamped shut and the lungs were carefully removed. A tube equipped with a two-way valve was inserted into the lower portion of the trachea, and the lungs were washed with 6 volumes (30 ml each) of 0.85% saline (at 37°C) containing 0.5 U of heparin per ml. The macrophage-rich fluid was pooled and centrifuged at $500 \times g$ for 15 min at 5°C. The macrophages were resuspended in attachment medium, and the cells were counted on a hemocytometer, using trypan blue exclusion as an indicator of cell viability. The cells were diluted to 2×10^6 macrophages per ml in the attachment medium composed of medium 199 supplemented with 100 μg of penicillin G per ml, 4 μg of gentamicin per ml, 100 mg of L-glutamine per liter, and 0.01% (wt/vol) bovine serum albumin (fraction V). One milliliter of the macrophage suspension was added to glass cover slips (22 by 22 mm) in small petri dishes (33 by 10 mm) and allowed to attach for 2 h at 37°C in 5% CO_2 in air. After attachment, the macrophages were washed, and fresh maintenance medium containing medium 199 supplemented with L-glutamine and 20% heat-inactivated rabbit serum was added. All macrophages were maintained in vitro overnight before being infected. Furthermore, samples of the macrophages were cultured on brain heart infusion agar to monitor for possible contamination by other bacteria. Using these methods, a normal rabbit lung yielded between 50×10^6 and 80×10^6 macrophages. We found that lungs from nonimmunized rabbits that yielded more than 100×10^6 macrophages were usually activated, and frequently they were contaminated with a virus (confirmed by electron microscopy), mycoplasma, or a gram-negative bacillus. When this happened, the macrophages were discarded.

Macrophages from immunized rabbits. Rabbits weighing 2 to 3 kg were injected with a heat-killed suspension of *N. asteroides* GUH-2 (1-week culture) in incomplete Freund adjuvant. Approximately 500 mg (wet weight) of cells in a total of 3.0 ml of suspension was administered subcutaneously behind the neck and intramuscularly in the hind leg. Two weeks later a second injection prepared in the same manner was given. A third injection was given in 2 additional weeks (a total of 6 weeks). During week 7, blood was drawn from the marginal ear vein, and the agglutinating antibody titer was determined against whole nocardial cell suspensions. The agglutinating antibody titer was usually 1:256 at this period of time after immunization (one rabbit out of ten had an antibody titer of 1:128). The immunized rabbits were then sacrificed, and the macrophages were obtained and maintained as described above. The numbers of macrophages obtained from the lungs of immunized rabbits were significantly greater than those obtained from the unmanipulated animals. Thus, between 250×10^6 and 400×10^6 macrophages per lung were obtained from immunized animals.

Infection of macrophages. A sample of the bacterial suspension (at each stage of growth) was diluted into maintenance medium as described previously (8) to give approximately 10^6 colony-forming units/ml. Dilutions of the bacterial suspensions were prepared and the actual colony-forming units per milliliter were determined. Two milliliters of the bacterial suspension in maintenance medium was added to the macrophage culture and incubated for 3 h. The suspension was removed from the macrophages, and they were washed three times with 1 ml of maintenance medium. These were added to the initial supernatant suspension, and the number of bacteria in the medium was determined (all determinations were carried out using five individual cover slips). At the same time five cover slips containing macrophages were lysed in distilled water for 15 min at 37°C. Dilutions of the lysed cell suspensions were plated to determine the number of macrophage-associated nocardial cells. Thus, the number of bacteria in the supernatant represented extracellular organisms, whereas the bacteria obtained from the washed cover slips represented bacteria that had been phagocytized by the macrophages. The total number of bacteria added to the macrophages was determined, and the sum of the supernatant and macrophage-associated organisms represented the percent recovered at 3 h after infection. Presumably, the difference between the total numbers of bacteria added and the total numbers recovered represented the cells that were inactivated by the macrophages. The percent phagocytosis represents the number of cells not recovered from the supernatant as compared to the original suspension. The quantitation of intracellular bacterial growth was determined as previously described (8, 9). Five cover slips from each time period were also prepared for light microscopy as previously described, and the numbers of macrophages per cover slip were determined by direct microscopic count. All experiments were repeated with at least three different rabbits from each group (nonimmune and immune) with similar results each time.

RESULTS

Cells of *N. asteroides* in the logarithmic phase of growth differ in their interactions with rabbit alveolar macrophages as compared to the cells in the stationary phase of the same culture. Light microscopy shows that cells of *N. asteroides* GUH-2 from all stages of growth are able to grow within alveolar macrophages obtained from nonimmunized rabbits (Fig. 1A, B, and C). Cultural data confirm that these cells are increasing in numbers; however, the organisms from the log phase grow more rapidly within macrophages than do cells obtained from the early (3 days) or late (7 days) stationary-phase cultures (cf Fig. 2, 3, and 4). By comparing the percentage of organisms phagocytized at 3 h with that remaining in the supernatant and by calculating total percent recovered as compared

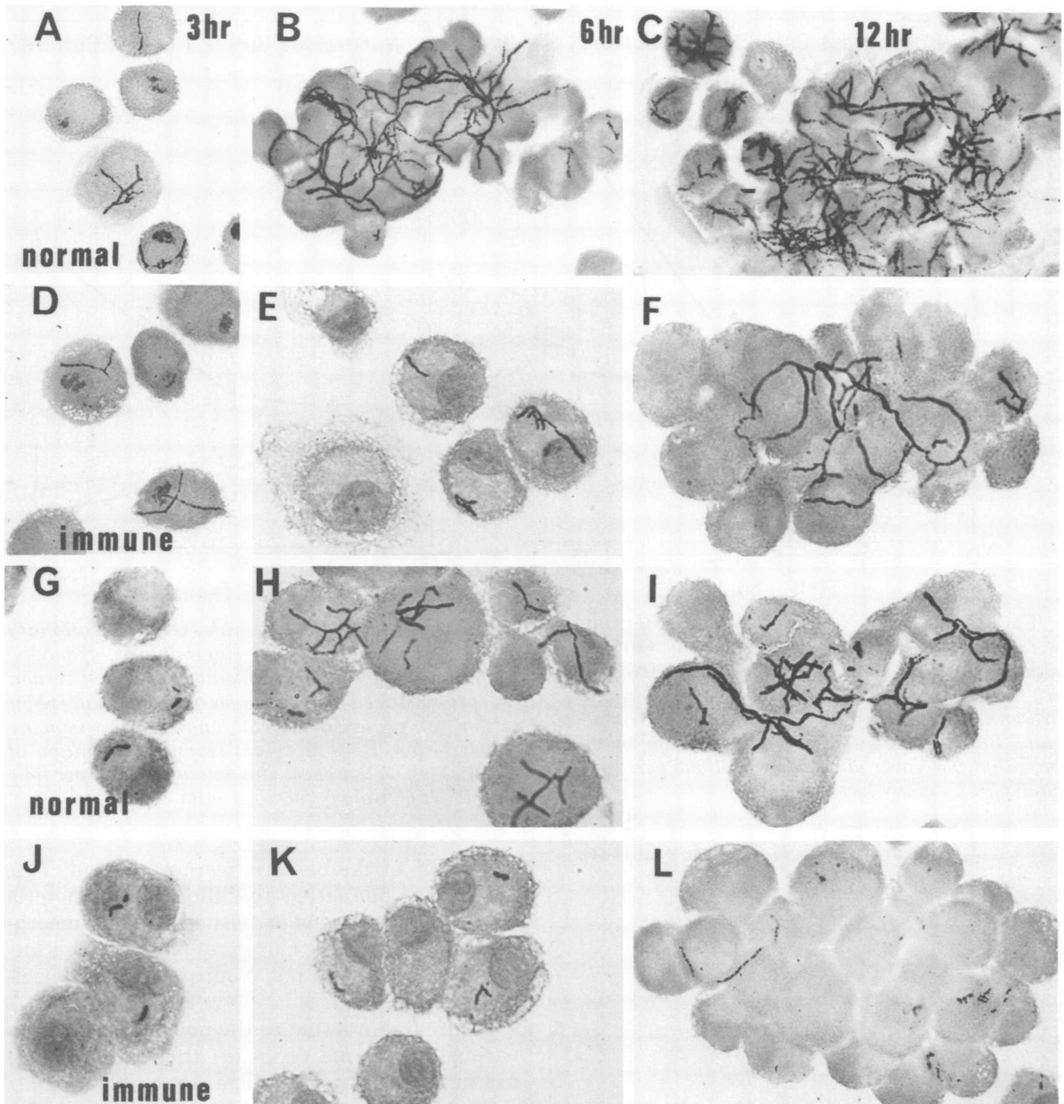

FIG. 1. *Light microscopic analysis of N. asteroides GUH-2 interactions with macrophages obtained from the lungs of normal and immunized rabbits. (A) Log-phase N. asteroides GUH-2 in normal alveolar macrophages at 3 h after infection. (B) Log-phase N. asteroides GUH-2 in normal macrophages at 6 h after infection. (C) Log-phase N. asteroides GUH-2 in normal macrophages at 12 h after infection. (D) Log-phase N. asteroides GUH-2 in macrophages obtained from immunized rabbits. The nocardial cells were preincubated for 1 h with immune serum. (E) is at 6 h whereas (F) is at 12 h after infection. (G) Stationary-phase N. asteroides GUH-2 as in (A). (H) Stationary-phase N. asteroides GUH-2 as in (B). (I) Stationary-phase N. asteroides GUH-2 as in (C). (J, K, L) Stationary-phase N. asteroides GUH-2 in macrophages from immunized rabbits as in (D), (E), and (F), respectively.*

to the inoculum, we evaluated uptake and killing of nocardial cells by macrophages. Table 1 presents the interactions of *N. asteroides* GUH-2 with normal rabbit alveolar macrophages in the presence of rabbit serum obtained from nonimmunized animals. There was a significant decrease in the macrophage's ability to phagocytize cells from the log phase of growth (16 h). Thus, approximately 5% of the viable log-phase cells were within alveolar macrophages at 3 h as compared to 30 to 40% of the stationary-phase bacteria. In addition, there appeared to be a 15% loss of viability in the log-phase culture as compared to the stationary-phase organisms, where

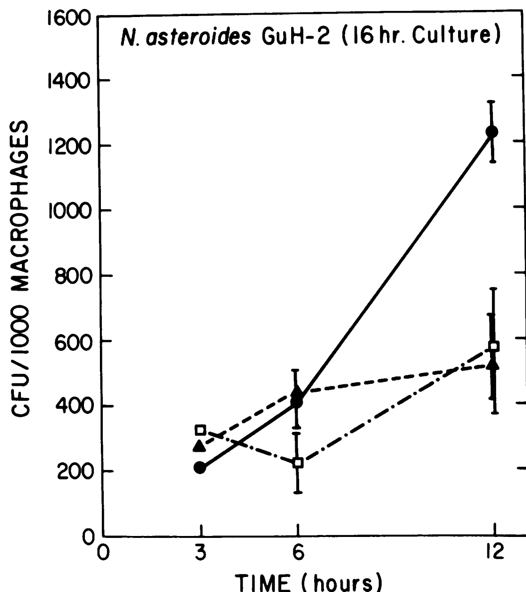

FIG. 2. *Growth of N. asteroides GUH-2 (log phase) in rabbit alveolar macrophages. Symbols: (●) normal macrophages plus normal rabbit serum; (▲) presensitized macrophages plus normal rabbit serum; (□) presensitized macrophages plus immune rabbit serum. Bars represent standard deviation of mean based on five cover slip determinations per time period. CFU, Colony-forming units.*

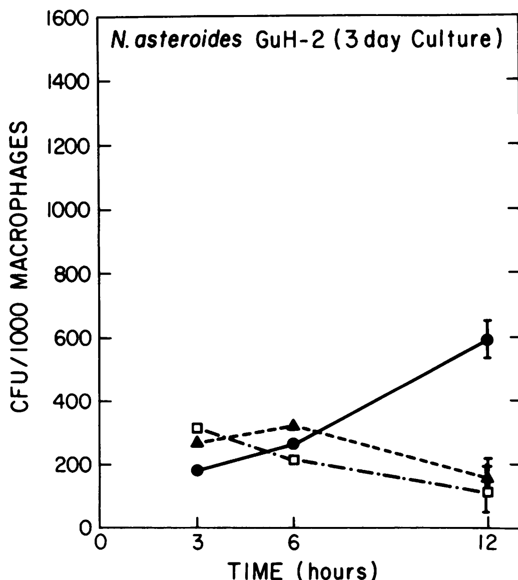

FIG. 3. *Growth of N. asteroides GUH-2 (early stationary phase) in rabbit alveolar macrophages. Symbols: (●) normal macrophages plus normal rabbit serum; (▲) presensitized macrophages plus normal rabbit serum; (□) presensitized macrophages plus immune rabbit serum. Bars represent standard deviation of mean based on five cover slip determinations per time period. CFU, Colony-forming units.*

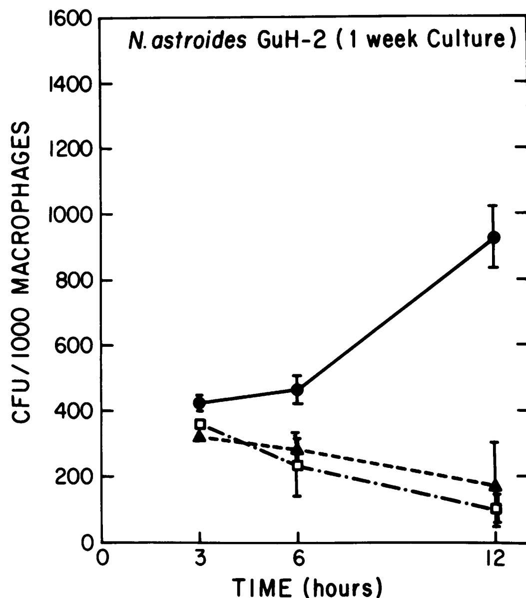

FIG. 4. *Growth of N. asteroides GUH-2 (stationary phase) in rabbit alveolar macrophages. Symbols: (●) normal macrophages plus normal rabbit serum; (▲) presensitized macrophages plus normal rabbit serum; (□) presensitized macrophages plus immune rabbit serum. Bars represent standard deviation of mean based on five cover slip determinations per time period. CFU, Colony-forming units.*

96 to 99% of the bacteria were recovered. This decrease is due either to killing of the bacteria by the macrophages or perhaps by the macrophages sequestering clumps of bacteria (this latter suggestion was not confirmed by light microscopy [Fig. 1]).

Figure 5 shows that as the nocardia grew from within alveolar macrophages, there was a loss in the numbers of macrophages that remained attached to the cover slips. An approximately 1:1 ratio of log-phase (16 h) cells of *Nocardia* to macrophages resulted in a 40% decrease in the number of cells that remained glass adherent 3 h after the addition of the bacteria. This reduction was significantly greater than that observed when the macrophages were infected with comparable amounts of either the 3-day or 1-week stationary-phase cells (Fig. 5). These data strongly suggest that log-phase cells of *N. asteroides* GUH-2 are initially more toxic for macrophages than similar amounts of stationary-phase bacteria.

We wished to determine whether specifically sensitized macrophages obtained from the rabbit lungs differed in their interaction with *N. asteroides*. Table 2 presents the interactions between the nocardial cells at different stages of growth and macrophages obtained from the lungs of

TABLE 1. *Nocardial interactions with normal rabbit alveolar macrophages plus control rabbit serum (3 h postinfection)*[a]

Age of nocardial culture	Viable nocardia in macrophages (%)	Total CFU[b] recovered (%)	CFU lost (%)	Total uptake by macrophages (viable and killed) (%)
16 h (log phase)	4.8 ± 0.1	84.9 ± 1.5	15.1 ± 1.0	19.9 ± 1.0
3 days (early stationary phase)	40.6 ± 5.0	99.4 ± 0.5	0.6 ± 0.4	41.2 ± 5.0
1 week (stationary phase)	32.2 ± 1.0	96.7 ± 1.0	3.3 ± 0.8	35.5 ± 1.8

[a] Mean ± standard deviation of the mean of three separate experimental determinations each involving five cover slip values.
[b] CFU, Colony-forming units.

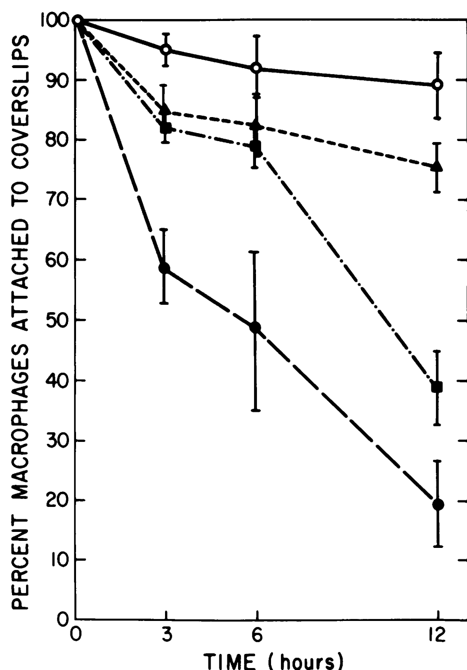

FIG. 5. *Toxicity of N. asteroides at different stages of growth for normal rabbit alveolar macrophages incubated with normal rabbit serum. Symbols: (●) N. asteroides GUH-2 log phase (16-h culture); initial infecting ratio of approximately 1:1 (nocardial cells/ macrophage). (■) N. asteroides GUH-2 early stationary phase (3-day culture); initial infecting ratio of approximately 5:1 (nocardial cells/macrophages). (▲) N. asteroides GUH-2 stationary phase (1-week culture); initial infecting ratio of approximately 10:1 (nocardial cells/macrophage). Note: The initital infecting ratios of nocardia to macrophages were adjusted in this experiment so that about the same nocardial cell mass for each time period was added to a given number of macrophages. (○) Uninfected, control macrophages. Bars represent standard deviation of mean based on five cover slip determinations per time period.*

rabbits that had been previously immunized against *N. asteroides* GUH-2. The data show that log-phase cells (16 h) at 3 h after addition to the macrophage culture had been affected the most. There was approximately a twofold greater inactivation of log-phase cells at 3 h after infection than observed with the normal macrophage population. Furthermore, more than twice the number of bacteria were phagocytized by the macrophages obtained from presensitized rabbits. For stationary-phase cultures there were slight increases in phagocytosis noted; however, there were significant decreases in percent recovery of the organisms, which indicated a greater ability of the macrophages obtained from presensitized rabbits to inactivate these nocardia (Table 2). The growth of *N. asteroides* in macrophages obtained from immunized rabbits is presented in Fig. 1, 2, 3, and 4. From these data it is clear that presensitized macrophages in the presence of normal rabbit serum can effectively retard the growth of stationary-phase cells of *N. asteroides* GUH-2 for at least 12 h (Fig. 3 and 4). There is a similar but less effective response towards the log-phase bacterial cells (Fig. 1 and 2). These latter cells were inhibited initially; however, a significant number were able to initiate intracellular growth within the macrophages (Fig. 1) and at 12 h after infection there were numerous microcolonies of *Nocardia* growing out of these macrophages. This was not observed at 12 h in the macrophages infected with stationary-phase bacteria (Fig. 1, 2, 3, and 4).

The experiments were repeated using macrophages from immunized rabbits; however, immune serum (not heat inactivated) was used to assess the role of complement and antibody on macrophage-nocardia interactions (Fig. 1, 2, 3, and 4). The results using stationary-phase organisms were virtually identical to those utilizing presensitized macrophages in the presence of normal (nonimmune) serum that had been heat inactivated (Table 3; cf. with Table 2). There appeared to be no enhanced uptake or killing brought about by the immune serum. This was in sharp contrast to the results when log-phase

TABLE 2. *Nocardial interactions with presensitized rabbit alveolar macrophages plus control rabbit serum (3 h postinfection)*[a]

Age of nocardial culture	Viable nocardia in macrophages (%)	Total CFU[b] recovered (%)	CFU lost (%)	Total uptake by macrophages (viable and killed) (%)
16 h (log phase)	12.6 ± 3.0	70.9 ± 4.0	29.1 ± 2.0	41.7 ± 5.0
3 days (early stationary phase)	32.1 ± 5.0	82.0 ± 1.5	18.0 ± 1.0	50.1 ± 6.0
1 week (stationary phase)	30.5 ± 2.0	80.1 ± 2.0	19.9 ± 1.0	50.4 ± 3.0

[a] Mean ± standard deviation of the mean of three separate experimental determinations each involving five cover slip values.
[b] CFU, Colony-forming units.

TABLE 3. *Nocardial interactions with presensitized rabbit alveolar macrophages plus immune rabbit serum (1/256 agglutinating antibody titer) 3 h postinfection*[a]

Age of nocardial culture	Viable nocardia in macrophages (%)	Total CFU[b] recovered (%)	CFU lost (%)	Total uptake by macrophages (viable and killed) (%)
16 h (log phase)	31.0 ± 5.0	60.5 ± 4.5	39.5 ± 3.0	70.5 ± 8.0
3 days (early stationary phase)	37.1 ± 4.0	84.0 ± 3.0	16.0 ± 2.5	53.1 ± 6.5
1 week (stationary phase)	31.5 ± 2.5	82.2 ± 2.5	17.8 ± 1.5	49.3 ± 4.0

[a] Mean ± standard deviation of the mean of three separate experimental determinations each involving five cover slip values.
[b] CFU, Colony-forming units.

(16 h) cultures were used (Table 3). There was enhanced phagocytosis and inactivation of the log-phase cells when immune serum was added to the culture (Table 3). Even though there was enhanced uptake and inactivation of the log-phase organisms, there was also intracellular growth of surviving bacteria, so that at 12 h after infection microcolonies of *N. asteroides* could be observed growing out of clumps of macrophages (Fig. 1F). These nocardial outgrowths were not observed in presensitized macrophages infected with stationary-phase cultures in the presence of immune serum (Fig. 1L).

DISCUSSION

The basic mechanisms of host defense against *N. asteroides* are not known. However, there is growing evidence that suggests that cell-mediated immunity (14, 15), T-cell lymphocytes (4, 5, 11), and macrophages are important components in host resistance to nocardial infections (2, 3, 8, 10, 12, 13, 16). The data presented herein suggest that normal alveolar macrophages present within the lungs of nonimmunized rabbits do not kill or significantly inhibit the growth of the virulent *N. asteroides* GUH-2 (regardless of culture age) upon initial contact. These data give in vitro support to the in vivo observations of *N. asteroides* GUH-2 reported previously (5). However, presensitized macrophages present within the lungs of immunized rabbits do effectively inhibit the growth of stationary-phase cells of *N. asteroides* GUH-2. Initially, the log-phase cells of this strain of nocardia are inactivated more effectively by presensitized macrophages than are the stationary-phase bacteria. However, a significant number of the surviving log-phase cells then initiate growth within these macrophages so that at 12 h microcolonies of nocardia are present within macrophage aggregates. This was not observed in the presensitized macrophages infected with stationary-phase cells.

It was previously shown that cells of *N. asteroides* GUH-2 in the logarithmic phase of growth are at least 1,000 times more virulent for mice than the 1-week stationary-phase organisms (7). The data presented above provide some possible reasons for the enhanced virulence. These include the following: (i) log-phase cells grow more rapidly within macrophages than do cells from stationary phase; (ii) the filamentous cells of log phase are not as easily phagocytized as the coccoid cells of stationary phase; (iii) log-phase cells are more toxic to macrophages than are comparable numbers of stationary-phase cells; and

(iv) the log-phase cells can grow within presensitized macrophages from immunized animals even in the presence of specific antibody more successfully than the stationary-phase cells, which are inhibited effectively by these macrophages. It is important to note, however, that serum obtained from immunized rabbits significantly enhanced initial uptake and inactivation of log-phase cells by presensitized macrophages, an effect that was not observed with the stationary-phase cells. These data suggest that antibody and humoral immunity may be important in protection of the host against the filamentous form of the organism.

It is important to note that *N. asteroides* GUH-2 is more virulent for mice than other strains such as *N. asteroides* 14759, which is of intermediate virulence, and the relatively avirulent *N. asteroides* 10905 (6). The interaction of these latter strains with rabbit alveolar macrophages has been reported previously (3, 8). It was found that *N. asteroides* 14759 was capable of replicating within macrophages, and the results were similar to those found with the more virulent *N. asteroides* GUH-2. The major differences between the two strains were that (i) initially more of the cells of *N. asteroides* 14759 were destroyed within the first 3 h of incubation (however, the surviving bacteria grew out of the macrophage aggregates similarly to that observed with *N. asteroides* GUH-2) and (ii) *N. asteroides* 14759 induced a more pronounced fusion of macrophages than did *N. asteroides* GUH-2. In contrast, cells of *N. asteroides* 10905 were either destroyed by rabbit alveolar macrophages or converted to transitional-phase, cell wall-deficient variants, indicating a removal of wall material by the macrophages. Later these L-phase variants grew within the macrophages, resulting in eventual destruction of them (8, 9).

The data presented above show a possible role of alveolar macrophages in host defense against pulmonary nocardiosis; however, the secondarily stimulated macrophages by themselves are probably not sufficient to control infection by virulent strains of *N. asteroides*. It has been shown clearly that T-cells are required (4, 5), and the roles of other host factors such as B-lymphocytes, humoral components, and polymorphonuclear phagocytes have not been adequately assessed (2).

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